

**Proliferative kidney disease in brown trout – infection level, pathology and mortality under field conditions**

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Running title: PKD related mortality and pathology under field conditions

**ABSTRACT:**

Proliferative kidney disease (PKD) is an emerging disease threatening wild salmonid populations. In temperature controlled aquaria, PKD can cause mortality rates of up to 85% in rainbow trout. So far, no data about PKD related mortality in wild brown trout are available. The aim of this study was to investigate mortality rates and pathology in brown trout kept in a cage within a natural river habitat which is known to harbor *Tetracapsuloides bryosalmonae*. Young of the year (YOY) brown trout, free of *T. bryosalmonae*, were exposed in the river Wutach, in the Northeast of Switzerland, during three summer months. Samples of wild brown trout caught by electrofishing near the cage location were examined in parallel. The incidence of PKD in cage exposed animals (69%) was not significantly different to the disease prevalence of wild fish, 82 and 80%. The mortality in cage exposed animals, however, was as low as 15%. At the termination of the exposure experiment, surviving fish showed histological lesions typical for PKD regression, suggesting that many YOY brown trout survive the initial infection. Our results at the river Wutach suggest that PKD in brown trout does not always result in high mortality under natural conditions.

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KEY WORDS: proliferative kidney disease • brown trout • mortality • pathology • temperature

## INTRODUCTION

Proliferative kidney disease (PKD) is an emerging disease of wild and farmed salmonid fish in Europe (Okamura et al. 2011). The disease is caused by the malacosporean parasite *Tetracapsuloides bryosalmonae* belonging to the Myxozoa (Hedrick et al. 1993, Canning et al. 2000, Okamura et al. 2001). The parasite's complex life cycle involves bryozoans as invertebrate (Anderson et al. 1999, Longshaw et al. 1999, Okamura et al. 2001) and salmonids as vertebrate hosts (Feist & Bucke 1993, Hedrick et al. 1993). *T. bryosalmonae* infects salmonids through skin and gills (Feist et al. 2001, Longshaw et al. 2002) or gills only (Morris et al. 2000, Grabner & El-Matbouli 2010), and is afterwards distributed systemically. The main target organ in the fish host is the kidney (Kent & Hedrick 1985), where it multiplies and differentiates from extrasporogonic stages in the renal interstitium to sporogonic stages in the lumen of renal tubuli (Kent & Hedrick 1985). Spores are then excreted via the urinary system (Kent & Hedrick 1985, Morris et al. 2002, Hedrick et al. 2004, Bettge et al. 2009). Transmission to bryozoans has been demonstrated in brown and brook trout (Morris & Adams 2006, Grabner & El - Matbouli 2008). In affected young-of-the-year (YOY) salmonids, proliferative and granulomatous nephritis and necrotizing vasculitis with thrombus formation has been described (Hedrick et al. 1993, El-Matbouli & Hoffmann 1994, Bettge et al. 2009). Surviving fish show chronic lesions with interstitial fibrosis and tubulonephrosis, followed by a complete regeneration of renal morphology (Schmidt-Posthaus et al. 2012, 2013). The spread and outcome of the infection are suspected to be enhanced by water temperature with severe disease and increased mortality at temperatures above 15°C (Wahli et al. 2008, Bettge et al. 2009, Okamura et al. 2011). In temperature controlled aquaria, PKD associated pathology and mortality are well documented in rainbow trout (*Oncorhynchus mykiss*, Bettge et al. 2009, Schmidt-Posthaus et al. 2012). These studies showed mortality up to 85% at a constant water temperature of 18°C. It was hypothesized that infection with *T. bryosalmonae* plays a significant role in the decline of wild salmonid populations, e.g. brown trout (*Salmo trutta fario*) in Switzerland and wild Atlantic salmon (*Salmo salar*) in Central

Norway (Wahli et al. 2002, Sterud et al. 2007, Krkošek et al. 2009). A widespread distribution of the disease in wild salmonid populations was also shown in other countries (Dash and Vasemägi 2014, Feist et al. 2002, Skovgaard and Buchmann 2012). However, so far, these studies only investigated prevalence, infection intensity and associated pathology under field conditions. Mortality rates and causes of mortality or contributing factors to it were not evaluated and are still under debate (Dash and Vasemägi 2014, Skovgaard and Buchmann 2012).

The aim of this study was to investigate PKD-related pathology and mortality in YOY brown trout exposed in a cage experiment to river water within their natural environment. Specifically, the following questions were investigated: (i) Does infection with *T. bryosalmonae* cause mortality in YOY brown trout under fluctuating temperature conditions as seen in the field?; and (ii) Does infection with *T. bryosalmonae* cause comparable pathology in cage exposed brown trout as seen in wild caught animals?

## **MATERIAL AND METHODS**

### **Study sites and fish sampling**

Experiments were conducted over a period of 3 months (16<sup>th</sup> of July to 8<sup>th</sup> of October 2013). The examined river system (Wutach) is situated in the southern part of Germany and has a 4.8 km border section to Switzerland in the midsection of the river. It passes through rural and urban areas before feeding into the larger river Rhine (Fig. 1). The Wutach has several tributaries; one of these was included in this study (Ehrenbach) (Fig. 1). Fish can migrate from the tributary into the Wutach but not vice versa because of an unpassable barrier. In former years, brown trout were regularly stocked in the Wutach (stocked fish originated from a trout hatchery nearby), but catches by anglers and electrofishing resulted in decreased numbers of fish in the midsection of the river (fishing statistics 1979-2013, unpublished data). Stocking was stopped one year prior to the beginning of the experiment. Therefore, investigated animals from the field sampling originated from natural spawning. In the tributary (Ehrenbach) included in this study, a stable brown trout population was recorded (fishing statistics 1979-2013, unpublished data) and no PKD records exist.

Investigations were conducted in two separate approaches, (i) a cage experiment was performed exposing YOY brown trout for three months to water in the Wutach; (ii) wild brown trout were sampled by electrofishing in two stretches in the Wutach and at one stretch in the Ehrenbach (field sampling). Water temperature was measured inside the cage and at two locations in river water, in the Wutach and the Ehrenbach.

#### *Cage experiment*

A cage, 1x1x0.5 m, was placed into the Wutach in the midstream part of the river (Fig. 1). Mesh size of the cage was 1 cm in diameter. An additional mesh was placed outside of the cage in the upstream position to avoid blocking of the cage by large floating refuses. This mesh was cleaned regularly.

During the experiment, the cage itself was not cleaned to avoid additional stress of the exposed animals. 100 YOY brown trout were purchased from a trout hatchery. Offspring of various broodstock, originally deriving from the Wutach, were mixed on the farm. Animals used in this experiment therefore originated from various mother animals. To determine if stocked fish were negative for infection with *T. bryosalmonae* at the beginning of the experiment, five brown trout were euthanized in clove oil and immediately examined as described below. Ten brown trout served as a negative control and were kept in spring water at the hatchery of origin.

In the middle of July 2013, 85 brown trout were placed into the cage. They were fed 1% of body weight with commercial fish food (TroCo Ultra, Coppens, Netherlands, pellet size 2 mm) using an automatic feeding machine ensuring continuous food supply. All fish were monitored daily for signs of disease or mortality. Moribund or dead fish were removed and investigated immediately. Three months after the start of the exposure experiment, all remaining trout were euthanized in clove oil, tagged and immediately investigated.

#### *Field sampling*

Fish were sampled at two stretches in the river Wutach, one stretch 2 to 3 km upstream of the cage experiment and one stretch 3 to 4 km downstream (Fig. 1). Additionally, the tributary was sampled a

few kilometres upstream of the entry into the river Wutach (Fig. 1). In September 2013, 10 and 11 YOY brown trout, respectively, were sampled at each of the two sites in the Wutach and 15 YOY brown trout in the Ehrenbach by electrofishing. Due to low density of YOY it was not possible to sample higher numbers of animals. Fish were euthanized separately in clove oil and examined as described below.

#### **Pathology, histopathology and immunohistochemistry**

Length of every dead or euthanized fish was recorded, followed by a complete necropsy. Animals originating from the cage experiment were weighted and the condition factor was calculated ( $100 \times \text{weight} / \text{length}^3$ , Bagenal, 1978). Macroscopic changes in the inner organs were recorded. Animals were then immediately fixed in 10 % buffered formalin for histopathological and immunohistochemical (IHC) examination. Kidney samples of all animals (cage experiment, field sampling) were routinely paraffin-embedded and 4  $\mu\text{m}$  sections were prepared for haematoxylin-eosin (H&E) stain. Suspicious cases were examined by IHC using a monoclonal anti-*Tetracapsuloides bryosalmonae* (PKX) antibody (AquaMAb-P01, Aquatic Diagnostics Ltd., Stirling, UK, Adams et al. 1992). Histopathological changes of the kidney were graded according to a common pathological grading system as 0 (no) to 6 (severe). Presence of *T. bryosalmonae* was examined on whole histological and IHC kidney sections and infection intensity was classified as 0 (no parasites present on whole slide), 1 (single parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a magnification of 200 to 400x.

#### **Real time PCR for detection of *T. bryosalmonae* DNA in kidney tissue**

Fish negative for *T. bryosalmonae* by histology and / or IHC were examined by real time PCR. Two 20  $\mu\text{m}$  sections of paraffin embedded material were deparaffinized. Lysis and extraction of total DNA was performed using DNeasy tissue Kit (Quiagen, Basel, Switzerland) according to the manufacturer's protocol. Samples were incubated with proteinase K at 56°C and 450 rpm overnight. The yield was

determined by spectrophotometry using the NanoDrop photometer (NanoDrop Technologies, Inc.,  
Wilmington, USA). Real time PCR was performed using SYBR GoTaq® qPCR Master Mix  
(Promega, Dübendorf, Switzerland) according to the manufacturer's instructions. Primer pair PKX3F  
(CTAAGTACATACTTCGGTAGA) and PKX4R (CCGTTACAACCTTGTTAGGAA), described by  
Kent et al. (1998), was used. A positive control sample obtained from the kidney of clinically infected  
brown trout from other studies and a negative control using water were included in the PCR  
procedure. A 297bp gene sequence of the small subunit ribosomal DNA (SSU rDNA) was detected.  
Samples with a threshold cycle value ( $C_t$ ) of 35 or lower and a melting temperature equal to the  
positive control were classified as positive. To confirm the specificity of the real time PCR, the real  
time PCR was repeated without dissociation stage and the PCR products were purified with  
NucleoSpint® (Machery-Nagel, Oensingen, Switzerland). The products were checked on a 1%  
agarose gel for amplification and molecular weight. To verify the sequence, the PCR products were  
sent to Microsynth AG, Balgach, Switzerland for sequencing. The identity of PCR products was  
determined by BLAST-n searching (Altschul et al, 1997) of the available sequences in the GenBank  
database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

## **Water temperature**

Water temperature was recorded every 2 hours by temperature loggers located inside the cage, in the  
Wutach near the cage, and in the Ehrenbach near the fish sampling site (Fig. 1). Mean values for each  
day were calculated and reported.

## **Statistics**

PKD prevalence was calculated as the sum of *T. bryosalmonae* positive animals per group divided by  
the total number of animals per group (percentage). The incidence of PKD describes the percentage of  
newly diseased individuals in the numerically defined group at risk for infestation over a limited time  
period. Significant differences between the groups were tested using the chi-square-test at a  $p \leq 0.05$   
significance level.

Parasite infection intensity was calculated by the sum of values for infection intensity per fish divided by the total number of infected animals. Mean of pathology score was calculated by the sum of pathology scores per fish divided by the total number of affected animals.

## RESULTS

### Water Temperature

In the Wutach, water temperature constantly exceeded 15°C for 38 days in a row from July to August (Fig. 1). Afterwards, water temperature exceeded 15°C over 10 additional non-consecutive days. As fish in the cage were exposed starting in the middle of July, when water temperature was already high, these animals experienced 39 days of water temperature above 15°C. In the Ehrenbach, water temperatures of 15°C and higher were only reached on 8 days, and no longer than 5 days in a row.

### Cage experiment

#### *Infection incidence, intensity, mortality and pathology*

Animals sampled at the beginning of the experiment before exposure to river water and those kept in the hatchery were in good body condition, and showed no signs of *T. bryosalmonae* infection. No *T. bryosalmonae* positive trout could be identified by real time PCR. No mortality was recorded in the group kept at the fish farm.

Inside the cage in the river Wutach, 15 brown trout died during the first 73 days (Fig. 2). Two animals which died during the first 14 days of the experiment showed neither macroscopical signs of PKD nor any histological kidney pathology nor presence of *T. bryosalmonae* DNA. In all animals that died at later time points, infection with *T. bryosalmonae* was evident. Parasites were visible histologically in nine animals. These nine animals showed moderate to severe kidney lesions typical for an acute PKD infection (mean value: 3.33, SD: 1.12, Fig. 3a). In the remaining four animals, autolysis was already advanced; thus in these fish, presence of parasite DNA could only be shown by real time PCR. No other signs of concurrent disease were detected in these spontaneous deaths, therefore mortality due to

PKD was calculated at 15% (13/85). These PKD related mortalities occurred between 45 and 73 days post exposure (p.e., Fig. 2).

The surviving 70 brown trout were euthanized after 84 days. Body condition was good (condition factor, mean value: 1.09, SD: 0.11). Thirty-four of the 70 surviving brown trout (49%) showed intact *T. bryosalmonae* by histology or IHC. Infection intensity in these animals varied between mild and moderate, at a mean intensity of 1.5 (SD: 0.65). By real time PCR, *T. bryosalmonae* DNA was detected in additional 12 brown trout.

Thirty-seven animals presented different pattern of kidney pathology. Sixteen trout showed acute renal changes with extrasporogonic parasites in the interstitium and intravascular thrombi composed of parasite stages, fibrin and mainly macrophages and few lymphocytes. Ten animals showed chronic active changes like interstitial fibrosis, tubulonephrosis and tubuloneogenesis together with an acute response to the disease as described above. In these animals, parasites were present in the interstitium, in the vessels, but also in the tubular lumen (Fig. 2d). However, detectable numbers of intratubular parasites were low. Six animals showed only solitary parasites in the interstitial tissue with only focal lesions. In five animals, kidney changes were already chronic (Fig. 3b,c) with spores in the tubular lumen (Fig. 3d) or no detectable parasites in vessels or the interstitial tissue. No signs of concomitant disease were present in any of the 70 animals.

Overall, PKD incidence was 69% (59 out of 85 exposed brown trout) after three months of exposure (Table 1), which was not significantly different to PKD prevalence in wild brown trout (see below).

## **Field sampling**

### *Infection prevalence, intensity and renal pathology*

In the Ehrenbach, none of the examined brown trout showed any signs of a *T. bryosalmonae* infection, neither by histology, immunohistochemistry nor by real time PCR. Kidney morphology was within normal limits in all examined fish.

In the Wutach, at the upstream and downstream location, PKD prevalence was 82% and 80%, respectively (Table 1). Infection intensity varied between mild and severe, with a mean value of 2.8



and 3.5, respectively (Table 1). At the upstream location, infection with *T. bryosalmonae* was mostly associated with moderate to severe acute kidney lesions (Fig. 3a, Table 1). The mean pathology score was 3.6 (Table 1). One animal showed acute renal lesions and an interstitial severe fibrosis, interpreted as a chronic active lesion. At the downstream location, all infected animals showed acute kidney lesions. The mean pathology score was 3.75. At the up- and at the downstream location, parasites were located in the interstitial tissue and in the vessels.

Chi-square analysis comparing PKD prevalence between the two field sampling groups up- and downstream of the cage and the PKD incidence in the cage showed no significant group differences ( $p=0.8921$ ).

## DISCUSSION

Previous studies hypothesized that increased PKD related mortality is a major contributor to the decline of brown trout populations (Wahli et al. 2008, Zimmerli et al. 2007, Schmidt-Posthaus et al. 2013), and YOY brown trout seemed to be especially affected (Burkhardt-Holm et al 2005, Hari et al. 2006). In several brown trout populations in Switzerland, lower YOY brown trout densities could be correlated to the occurrence of PKD (Borsuk et al. 2006). In other countries (e.g. Denmark, Norway), the effect of *T. bryosalmonae* infections on the population level is still under discussion (Skovgaard and Buchmann 2012, Mo et al. 2011). *T. bryosalmonae* positive brown trout were found in high numbers in different fish populations by PCR, however clinical signs were only rare (Skovgaard and Buchmann 2012, Mo et al. 2011). Therefore, the authors discussed that *T. bryosalmonae* infections are not always detrimental for wild fish. However, mortality rates related to particular diseases in wild fish populations are difficult to measure as usually no post-mortem data can be collected. Therefore, the aim of this study was to evaluate PKD related pathology and mortality in YOY brown trout kept in a cage in the river Wutach. Interestingly, mortality stayed as low as 15% in our experiment, despite the fact that 69% of caged fish became infected during the experimental period. In temperature controlled aquaria using rainbow trout, mortality up to 85% was shown at constant water temperatures of 18°C (Schmidt-Posthaus et al 2012) over a time period of 75 days post exposure. After 47 days p.e.,

cumulative mortality already reached 77% (Bettge et al. 2009). In our cage experiment, PKD related mortality in brown trout occurred between 45 and 73 days p.e. (Fig. 2). Therefore, onset of mortality was delayed compared to laboratory experiments using rainbow trout (Bettge et al. 2009). Species differences could be an explanation for this difference with brown trout being less sensitive to the infection. Water temperature higher than 15°C is thought to be critical for disease related mortality (Bettge et al. 2009, Okamura et al. 2011). In our experiment, daily mean water temperature exceeded 15°C for 39 days inside the cage. Fish were exposed relatively late, starting middle of July, whereas native YOY trout are exposed much earlier to infestation. Low mortality could therefore also be due to insufficient infection rate due to low spore density in the water. Spore release by bryozoan seems to be undulating instead of continuous with a peak value in June / July (Hanna Hartikainen, pers. comm.). Further research is needed to investigate this possible impact, e.g. by exposing brown trout in river water over the whole season with regular samplings during the exposition period to monitor disease development in exposed brown trout. However, incidence of infected animals at the end of our experiment was 69%, similar ( $p>0.05$ ) to the disease prevalence found in wild brown trout at the nearby locations in the Wutach (80 and 82%). Additionally, lesions in caged fish were comparable to those occurring in wild fish in the same river during the time period of the experiment, severity of pathology was advanced in wild fish compared to caged animals. However, whereas wild brown trout and caged brown trout dying during the experiment showed acute kidney changes with intravascular extrasporogonic parasites, as well as parasites in the renal interstitium, caged fish sampled at the end of the experiment showed a high percentage (41%) of chronic or chronic active lesions with spores already present inside renal tubuli. This indicates spore translocation into the tubular lumen (sporogonic stage) and spore excretion. The low number of detectable spores in the tubular lumen by immunohistochemistry can be due to the fact that the used monoclonal antibody (anti-*Tetracapsuloides bryosalmonae* (PKX) antibody, AquaMAB-P01, Aquatic Diagnostics Ltd., Stirling, UK) only partly detects sporogonic stages of the parasite, and therefore the number of intratubular spores was probably underestimated. Between the sampling of wild fish and the end of the cage experiment, there was a time delay of 4 weeks, which might explain

this morphological difference with more chronic stages in caged animals. Therefore, these animals seemed to be survivors, already within a recovery stage of the disease. Unlike rainbow trout, brown trout are able to excrete intact spores which can re-infect bryozoa (Morris & Adams 2006, Grabner & El - Matbouli 2008). In the field, additional stressors, like concurrent diseases, can retard parasite development in the kidney and the kidney regeneration process in wild brown trout (Schmidt-Posthaus et al. 2013). Food competition can also be regarded as one additional stressor in the field. This stressor was not present in our study as animals in the cage were fed with artificial food which was also reflected by the good body condition of caged brown trout.

Our results suggest that PKD in brown trout does not always result in high mortality under natural conditions. Additional research is needed to confirm these indications and to investigate possible applications on other river systems.

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369 **List of figures**

370 Fig. 1: a. Location of field sampling sites (grey bars), cage (black rectangle) and location of  
371 temperature logger in the Wutach and the Ehrenbach (grey triangles). Black bars indicate barriers  
372 which are not traversable by brown trout. b. Temperature curve in the Ehrenbach. c. Temperature  
373 curve in the Wutach in the cage. Thin lines indicate 15°C which was shown to be critical for PKD  
374 related clinical signs and mortality in trout. Thick dark line indicates period where temperature stayed  
375 above 15°C.

376

377 Fig. 2: Survivorship curve of brown trout (*Salmo trutta fario*) kept in the cage in the river Wutach.

378 P.e.= post exposure in river water

379

380 Fig. 3: Histological lesions found in brown trout kept in the cage in river water, a. acute renal change  
381 with thrombus formation (arrow), thrombus consists of mainly macrophages, fewer lymphocytes and  
382 parasites (open arrowhead), b. chronic renal changes with fibrosis around tubuli and vessels (open  
383 arrowheads), c. higher magnification of chronic changes (star) with fibrosis and infiltration with  
384 macrophages and eosinophilic granular cells, HE stain. d. IHC investigation showing positive parasite  
385 spores (arrowhead) in the tubular lumen. Bars (a,c) = 25 µm, (b) = 50 µm, (d) = 10 µm

## List of tables

Table 1: *Salmo trutta fario*, field sampling and cage experiment, different locations of sampling, length, prevalence of infected brown trout, infection intensity (shown as mean values  $\pm$  standard deviation) and associated renal pathology (shown as mean values  $\pm$  standard deviation), PKD related mortality, nk = not known. Histopathological changes of the kidney were graded as 0 (no), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe) or 6 (severe). Presence of *T. bryosalmonae* was classified as 0 (no parasites present on whole slide), 1 (single parasites), 2 (mild infection rate), 3 (mild to moderate infection rate), 4 (moderate infection rate), 5 (moderate to severe infection rate) or 6 (severe infection rate) at a magnification of 200 to 400x.

Location			<i>T.b. in brown trout</i>					
			n	Length (cm) (Mean $\pm$ SD)	Prevalence (%)	Infection intensity (Mean $\pm$ SD)	Pathology (Mean $\pm$ SD)	Mortality (%)
Field sampling  Cage experiment	Wutach	Upstream location	11	10.2 $\pm$ 1.0	82	2.8 $\pm$ 1.6	3.6 $\pm$ 1.7	nk
		Downstream location	10	10.4 $\pm$ 0.8	80	3.5 $\pm$ 2.1	3.75 $\pm$ 1.7	nk
		Ehrenbach	15	9.3 $\pm$ 1.5	0	0 $\pm$ 0	0 $\pm$ 0	nk
	Fish farm	Starting control	5	8.8 $\pm$ 0.8	0	0 $\pm$ 0	0 $\pm$ 0	0
	Fish farm	Negative control	10	11.7 $\pm$ 1.8	0	0 $\pm$ 0	0 $\pm$ 0	0
	Wutach	Caged animals	85	11.2 $\pm$ 1.3	69	1.6 $\pm$ 1.1	2.3 $\pm$ 1.4	15